American Journal of Hematology 56:239–243 (1997)

Dissection of the Association Status of Two Polymorphisms in the β-Globin Gene Cluster With Variations in F-Cell Number in Non-Anemic Individuals

Taha Merghoub,[1] Bruno Perichon,[1] Micheline Maier-Redelsperger,[1,2] Salvatore Pietro Dibenedetto,[3] Piera Samperi,[3] Rolande Ducrocq,[1] Nicole Feingold,[4] Jacques Elion,[1] Gino Schiliro,[3] Dominique Labie,[5] and Rajagopal Krishnamoorthy[1]*

[1]INSERM U 458, Hôpital Robert Debré, Paris, France
[2]Laboratoire d'Hématologie, Hôpital Tenon, Paris, France
[3]Divisione Di Hematologia ed Oncologia Pediatrica, Universita Di Catania, Catania, Italy
[4]INSERM U155, Université Denis Diderot-Paris VII, Paris, France
[5]ICGM, CHU Cochin, Paris, France

Expression of fetal hemoglobin (Hb F) is under polygenic control involving determinants both linked and unlinked to the β-globin gene cluster on chromosome 11. Variations in the DNase I-hypersensitive site 2 of the locus control region (LCR-HS2) and a C → T change at position −158 from the Gγ-gene (detected as an *Xmn*I polymorphism) correlate with the high level of Hb F expression in patients with sickle-cell anemia and β-thalassemia. Interpretation of data under these conditions of anemic stress is difficult because the preferential survival of Hb F-containing erythrocytes (F-cells) may not reflect the true status of Hb F expression. We investigated the relationship between these markers and Hb F expression in terms of F-cell levels in 48 unrelated non-anemic AS heterozygotes from Sicily. The β^S-chromosome of all these individuals was of the Benin haplotype and they differed only by their β^A chromosomes. We demonstrate that F-cell expression is more strongly associated with LCR-HS2 polymorphism than with *Xmn*I polymorphism. The observed association between *Xmn*I polymorphism and Hb F expression is very likely to be due to linkage disequilibrium with LCR-HS2 sequences. Am. J. Hematol. 56:239–243, 1997. **© 1997 Wiley-Liss, Inc.**

Key words: fetal hemoglobin; F-cells; polymorphism; locus control region

INTRODUCTION

In normal adults, synthesis of fetal hemoglobin (Hb F) persists at a very low level (<1%), and is restricted to a subpopulation of erythrocytes termed ''F-cells'' [1,2]. The genetic conditions associated with increased Hb F levels in adult life are described as hereditary persistence of fetal hemoglobin (HPFH) [3]. Some rare forms of HPFH, with 20–30% Hb F in heterozygotes, exhibit a clear Mendelian inheritance pattern and are caused by extensive deletions within the β-globin gene cluster or point mutations in the promoter region of one of the two fetal globin genes [3,4]. However, the most common form of HPFH in normal adults is characterized both by a moderate increase in Hb F (in the 1 to 5% range), and by its uneven distribution in red cells (heterocellular HPFH) [5]. This phenotypic condition exhibits genetic heterogeneity. In some families, the genetic determinant behaves as an allele of the β-globin gene complex whereas in others, it is unlinked to this region of chromosome 11 [6–14].

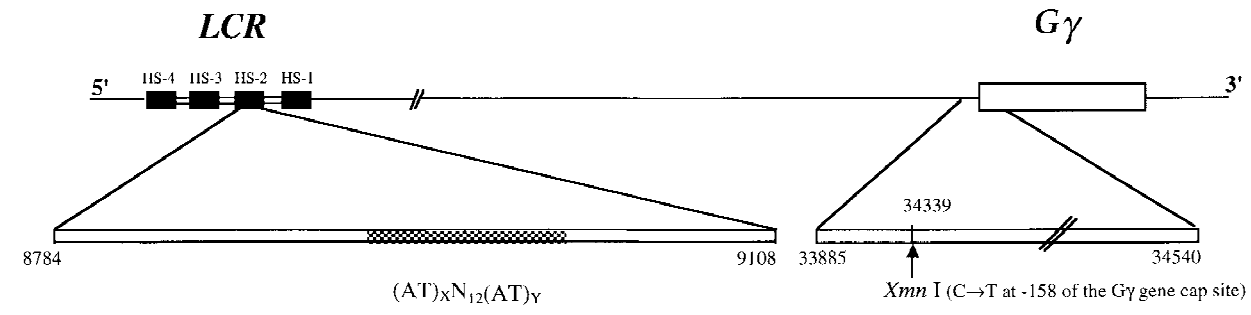
There are two markers of the β-globin gene complex associated with a modest rise in Hb F levels. One is a

© 1997 Wiley-Liss, Inc.

Contract grant sponsor: European Union; Contract grant number: TS3-CT93-0244 DG12HSMU; Contract grant sponsor: Progetto Tallasemia; Contract grant numbers: T/25, T2/22, T3/99; Contract grant sponsor: Societé Française d'Hematologie.

*Correspondence to: Rajagopal Krishnamoorthy, INSERM U 458, Hôpital Robert Debré, 48 bd Sérurier, 75019 Paris, France. E-mail: krishna@infobiogen.fr

Received for publication 16 December 1996; Accepted 9 July 1997



A	$(AT)_8N_{12}GT(AT)_7$	-	all 48 βˢ chromosomes
			βᴬ chromosomes
B	$(AT)_8N_{12}(AT)_{11}$	-	4
C	$(AT)_8N_{12}(AT)_{12}$	-	1
D	$(AT)_9N_{12}(AT)_{10}$	+	11
E	$(AT)_9N_{12}(AT)_{11}$	-	6
F	$(AT)_9N_{12}(AT)_{12}$	-	7
G	$(AT)_{10}N_{12}(AT)_{12}$	-	6
R (reference)	$(AT)_{10}N_{12}(AT)_{11}$	-	11
D*	$(AT)_9N_{12}(AT)_{10}$	-	2
G*	$(AT)_{10}N_{12}(AT)_{12}$	+	1
R*	$(AT)_{10}N_{12}(AT)_{11}$	+	1

* see the text

Fig. 1. LCR-HS2 sequence configurations and *Xmn*I status of 48 AS individuals from South East Sicily. The configurations of LCR-HS2 are designated A to G, and R is the reference sequence configuration initially registered as the HUMHBB reference sequence (accession number: U01317). Enlarged segments correspond to the regions amplified by PCR. Nucleotide numbering is according to the HUMHBB sequence. Configuration A of LCR-HS2 was found exclusively on β^S-chromosomes typical of the Benin haplotype. All other configurations are associated with β^A-chromosomes.

common C → T variation at position −158 upstream from the Gγ gene cap site (detected as an *Xmn*I restriction site polymorphism) [15–17]. The other is a set of sequence variations in the 5′ hypersensitive site (HS2) of the β-globin locus control region (LCR) [18–20]. The presence of the −158 Gγ *Xmn*I site is consistently associated with high Gγ expression but inconsistently with elevated Hb F expression in sickle cell anemia (SCA) and β-thalassemia [21]. Its association with a modest rise in F-cell number in hematologically normal individuals has also been reported [17]. Similarly, a specific sequence configuration of the LCR-HS2 region is associated with increased levels of Hb F in patients with sickle-cell anemia and β-thalassemia [18,19]. We have shown that the same LCR-HS2 sequence configuration (which we designated as ''configuration D'') is associated with both higher levels of Hb F and a high percentage of F-cells in non-anemic individuals [20].

In this study, we examined possible independent and/or combined effects of the *Xmn*I and LCR-HS2 polymorphisms on expression of F-cells in the same group of 48 unrelated non-anemic AS heterozygotes. We studied F cell levels rather than Hb F levels because measurement of Hb F in non-anemic individuals with low levels of Hb F expression is prone to errors [17]. Our data show that LCR-HS2 configuration D has a stronger association with F-cell numbers than does the *Xmn*I polymorphism. Thus, the reported association of the *Xmn*I site with increased Hb F expression [17] both in anemic and non-anemic conditions is likely to be due to its linkage disequilibrium with LCR-HS2 configuration D.

METHODS

Blood samples were collected from members of 15 families from South East Sicily, selected through an affected family member with sickle cell disease. Hematological data were obtained using an automated cell counter. The Hb S/Hb A ratio was determined by a sensitive HPLC method [22]. The F-cell assay was performed by microscopy with an immunofluorescence method using a monoclonal antibody raised against human γ-globin chains [23]. Analysis of the classical β-globin gene cluster RFLP-haplotypes was performed using a PCR-based approach [24]. The nucleotide sequence of the LCR-HS2 region was determined as described earlier including an allele-specific sequencing procedure [20,22–25]. Forty-eight unrelated non-anemic AS heterozygotes (males = 23, females = 25), with a mean age of 44 years (range = 25 to 70), were selected from the 15 families for the study. The relationship between F-cell number and the genetic markers (Fig. 1) in these indi-

viduals was investigated by statistical comparison using the Student's *t*-test.

RESULTS

Haplotype analysis of DNA from the homozygote SS patients from Sicily showed that all the β^S-chromosomes in these families carry the ''Benin'' haplotype (haplotype 19) and the LCR-HS2 $(AT)_8N_{12}GT(AT)_7$ configuration typical of this haplotype (here designated as configuration A) [25]. This is consistent with previous studies [26]. Thus, all 48 AS individuals studied share an identical β^S-chromosome. Family studies made the unambiguous phasing of the polymorphic markers along their β^A-chromosome possible. Seven different configurations of the LCR-HS2 $(AT)_xN_{12}(AT)_y$ motif in all were found associated with the β^A-chromosomes (Fig. 1). They were assigned letters from B to G and the reference sequence (HUMHBB) was designated R. Configuration D was the most common in our series (13 of 48 chromosomes). This is the configuration initially described for β^S-chromosomes of the ''Senegal'' type (haplotype 3) [25]. Configuration R was the second most common (11 of 48).

The presence of both the *Xmn*I site and configuration D of the LCR-HS2 was detected in 11 β^A-chromosomes (Fig. 1). Seven different configurations of the LCR-HS2 sequence, including configuration D (n = 2) (designated D* in Fig. 1), were found in chromosomes where the −158 Gγ *Xmn*I site was absent (n = 35). Only two chromosomes bearing LCR-HS2 sequence configurations other than D (configuration G* and R* in Fig. 1) also had the *Xmn*I site. Thus, association between *Xmn*I (+) site and configuration D was not constant, as shown by the existence of two other combinations [*Xmn*I (−)/configuration D and *Xmn*I (+)/non D configuration].

Hematological indices and A/S ratios indicated that none of the 48 individuals studied was anemic or a carrier for α-thalassemia (data not shown). As the β^S-chromosomes of these AS individuals were identical, it was possible to specifically assess the association between F-cell levels and the status of the LCR-HS2/*Xmn*I polymorphisms of their β^A-chromosome. Individuals with the *Xmn*I site or LCR-HS2 configuration D on their β^A-chromosome had a higher mean F-cell level than those lacking these sequences and this difference was statistically significant (Table IA). However, the significance was higher for LCR-HS2 configuration D rather than for the *Xmn*I site (Table IB).

Four subjects did not simultaneously have the *Xmn*I site and LCR-HS2 configuration D on the same β^A-chromosome. Two had the *Xmn*I site but not configuration D. The F-cell percentage in these two individuals was lower than those with configuration D (5.1 ± 1.7 vs. 8.3 ± 5.4). The other two individuals who had configuration D but not the *Xmn*I site had a higher F-cell percentage than those with other LCR-HS2 sequence configurations (10.1 ± 4.07 vs. 4.6 ± 3.8). These data are consistent with a stronger association of F-cell level with LCR-HS2 status than with the *Xmn*I polymorphism.

TABLE I. Effect of the −158 Gγ (Detected by *Xmn*I Restriction Site) and LCR-HS2 Polymorphisms on F-Cell Levels in 48 Unrelated AS Heterozygotes

A. −158 Gγ C/T polymorphism	LCR HS2 polymorphism	n	F cell (%)
*Xmn*I (+)	Any configuration	13	7.8 ± 5.2
	Configuration D (Senegal type)	11	8.3 ± 5.4
	Non D configuration	2	5.1 ± 1.7
*Xmn*I (–)	Any configuration	35	4.9 ± 3.9
	Configuration D (Senegal type)	2	10.1 ± 4.1
	Non D configuration	33	4.6 ± 3.8

B.	F-cell (%)
*Xmn*I	
+	7.8 ± 5.1
–	4.9 ± 3.9
P value (*t*-test)	0.04
5′ LCR-HS2	
D	8.6 ± 5.1
Others	4.6 ± 3.6
P value (*t*-test)	0.0046

DISCUSSION

Variations in Hb F and F-cell level have mostly been studied in SCA patients, i.e., in a situation of erythropoietic stress. Association studies have shown that patients with the ''Senegal'' (haplotype 3) or ''Indian'' (haplotype 31) β^S-haplotypes, both of which have the *Xmn*I site, have higher mean Hb F levels than the others but exceptions are not uncommon.

One SCA patient homozygous for the ''Benin'' haplotype (no *Xmn*I site) was found to have an unusually high Hb F level. The LCR-HS2 sequence of this patient was that usually associated with the ''Senegal'' β^S haplotype and not with the ''Benin'' β^S haplotype. This observation suggested a possible association between Hb F levels and polymorphism of the LCR-HS2 region, specifically between the ''Senegal'' type LCR-HS2 and higher levels of Hb F expression [18]. Similar observations have also been made for β-thalassemia [19]. In both cases, patients were under anemic stress and only Hb F level was measured, not levels of F-cells. Interpretation of the data is difficult because the high peripheral level of Hb F may reflect either higher production of F-cells per se or selective survival of Hb F-containing erythrocytes.

We studied non-anemic individuals whose peripheral levels of Hb F should reflect essentially the rate of F-cell

production, thus avoiding problems associated with the presence of anemic stress. The family study of these unrelated AS individuals, each carrying an identical set of sequences in the β-globin gene cluster on one chromosome (β^S-chromosome) and varying sequences on the other (β^A-chromosome), made it possible to study Hb F levels exclusively in relation to the polymorphic pattern of the β^A chromosome.

Among the two polymorphic markers of the β-globin gene cluster associated with Hb F and F-cell status, the LCR-HS2 polymorphism was a better predictive marker of the percentage of F-cells than the *Xmn*I polymorphism. Thus, the previously reported association between the *Xmn*I polymorphism and the F-cell percentage may be due to the linked LCR-HS2 sequence variations. In this study, the dissection of the individual effects of LCR-HS2 and *Xmn*I polymorphisms was made possible by the availability of some novel combinations of these two polymorphisms in β^A chromosomes, which is rare in studies involving patients homozygous for sickle-cell anemia or β-thalassemia. Interestingly, β^A chromosomes bearing the LCR-HS2 sequence configuration (configuration G) identical to that associated with Indian β^S haplotype did not exhibit any increase in F-cell level. The mechanism by which Hb F levels increase in Indian sickle-cell patients might be different.

It was proposed that the variable production of Hb F under anemic stress might be related to changes at 3 nucleotide positions (8580, 8598, and 9114) flanking the polymorphic LCR-HS2 simple sequence repeat [18]. Indeed, these nucleotides are located within sequences that exhibit high homology to the enhancer sequence of the Friend's murine/Moloney leukemia virus [27], the Sp1 binding site [28], and the TEF-2 (CACCC box protein, or CBP) binding site [29], respectively. Our data do not support this hypothesis because nucleotides at these three positions were identical in our samples. The differences, in our sample, are restricted to the (AT)xN12(AT)y repeat motif. Hence, it is possible that sequence variations in the motif itself might be directly or indirectly involved in Hb F expression. Similar AT repeat sequences in the 5′ flanking region of the β-globin gene are also polymorphic. This region has a sequence-dependent differential binding affinity for a nuclear protein BP1 (β-protein 1) [30]. It was postulated that the binding of BP1 to this region may down-regulate β-globin gene transcription.

We recently found that the AT repeat sequences from both the LCR-HS2 region and the β-globin gene promoter compete for a common nuclear protein (presumably BP1) in a gel mobility shift assay (data not shown). Thus, involvement of this polymorphic region in the regulation of Hb F expression cannot be ruled out. However, we cannot totally exclude the possibility that these polymorphic sequences are markers for variations in a yet to be identified regulatory region critical to γ-gene expression.

ACKNOWLEDGMENTS

This work was supported by grants from the European Union (TS3-CT93-0244 DG12HSMU), and from Progetto Tallasemia T/25, T2/22, and T3/99 from the Sicily region. T.M. is the recipient of a fellowship from the ''Société Française d'Hématologie''

REFERENCES

1. Boyer SH, Belding TK, Margolet L, Noyes AN: Fetal hemoglobin restriction to a few erythrocytes (F-cells) in normal human adults. Science 188:361–363, 1975.
2. Wood WG, Stamatoyannopoulos G, Lim G, Nute PE: F-cells in the adult: Normal values and levels in individuals with hereditary and acquired elevations of Hb F. Blood 46:671–682, 1975.
3. Stamatoyannopoulos G, Nienhuis AW: Hemoglobin switching. In Stamatoyannopoulos G, Nienhuis AW, Majerus PW, Varmus H (eds): ''The Molecular Basis of Blood Diseases.'' Philadelphia: W.B. Saunders Company, 1994, pp 107–136.
4. Wood WG: Increased Hb F in adult life. In Higgs DR, Weatherall DJ (eds): ''The Hemoglobinopathies.'' London: Baillière Tindall, 1993, pp 177–213.
5. Marti HR, Butler R: Hämoglobin F und Hämoglobin A_2-Vermehrung bei der Schweizer Bevölkerung. Acta Haematol 26:65–74, 1961.
6. Wood WG, Weathearall DJ, Clegg JB: Interaction of heterocellular hereditary persistence of fœtal hemoglobin with β-thalassaemia and sickle cell anaemia. Nature 264:247–249, 1976.
7. Zago MA, Wood WG, Clegg JB, Weatherall DJ, O'Sullivan M, Gunson H: Genetic control of F-cells in human adults. Blood 53:977–986, 1979.
8. Old JM, Ayyub H, Wood WG: Linkage analysis of nondeletion hereditary persistence of fetal hemoglobin. Science 215:981–982, 1982.
9. Gianni AM, Bregni M, Cappellini MD, Fiorelli G, Taramelli R, Giglioni B, Comi P, Ottolenghi S: A gene controlling fetal hemoglobin expression in adults is not linked to the non-α globin cluster. EMBO J 2:921–925, 1983.
10. Miyoshi M, Kaneto Y, Kawai H, Ohchi H, Niki S, Hasegawa K, Shirakami A, Yamano T: X-linked dominant control of F-cells in normal adult life: Characterization of the Swiss type as hereditary persistence of fetal hemoglobin regulated dominantly by gene(s) on X-chromosome. Blood 72:1854–1860, 1988.
11. Martinez G, Novelletto A, Di Rienzo A, Felicetti L, Colombo B: A case of hereditary persistence of fetal hemoglobin caused by a gene not linked to the β-globin cluster. Hum Genet 82:335–337, 1989.
12. Chang YC, Smith KD, Moore RD, Serjeant GR, Dover GJ: An analysis of fetal hemoglobin variation in sickle cell disease: The relative contributions of X-linked factor, β-globin haplotypes, α-globin gene number, gender and age. Blood 85:1111–1117, 1994.
13. Thein SL, Sampietro M, Rohde K, Rochette J, Weatherall DJ, Lathrop GM, Demenais F: Detection of a major gene for heterocellular hereditary persistence of fetal hemoglobin after accounting for genetic modifiers. Am J Hum Genet 54:214–228, 1994.
14. Craig JE, Rochette J, Fisher CA, Weatherall DJ, Marc S, Lathrop GM, Demenais F, Thein SL: Dissecting the loci controlling fetal hemoglobin production on chromosome 11 p and 6 q by the regressive approach. Nature Genet 12:58–64, 1996.
15. Gilman JG, Huisman THJ: DNA sequence variation associated with elevated Gγ-globin production. Blood 66:783–787, 1985.
16. Labie D, Dunda-Belkhodja O, Rouabhi F, Pagnier J, Ragusa A, Nagel

RL: The −158 site 5′ to the Gγ-gene and Gγ expression. Blood 66: 1463–1465, 1985.
17. Sampietro M, Thein SL, Contreras M, Pazmany L: Variation of Hb F and F-cell number with the Gγ *Xmn*I (C → T) polymorphism in normal individuals. Blood 79:832–833, 1992.
18. Öner C, Dimovski AJ, Altay C, Gurgey A, Gu YC, Huisman THJ, Lanclos KD: Sequence variations in the 5′ hypersensitive site-2 of the locus control region of β^S chromosomes are associated with different levels of fetal globin in hemoglobin S homozygotes. Blood 79:813–819, 1992.
19. Beris P, Kitundu MN, Baysal E, Öner C, Lanclos KD, Dimovski AJ, Kutlar F, Huisman THJ: Black β-thalassemia homozygotes with specific sequence variations in the 5′ hypersensitive site-2 of the locus control region have high levels of fetal hemoglobin. Am J Hematol 41:97–101, 1992.
20. Merghoub T, Périchon B, Maier-Redelsperger M, Labie D, Feingold N, Dibenedetto, SP, Samperi P, Schiliro G, Ducrocq R, Elion J, Krishnamoorthy R: Variation of fetal hemoglobin and F-cell number with the LCR-HS2 polymorphism in nonanemic individuals. Blood 87:2607–2609, 1996.
21. Labie D, Pagnier J, Lapoumeroulie C, Rouahbi F, Dunda-Belkhodja O, Chardin P, Beldjord C, Wacjman H, Fabry ME, and Nagel RL: Common haplotype dependence of high Gγ-globin gene expression and high Hb F levels in β-thalassemia and sickle cell anemia patients. Proc Natl Acad Sci USA 82:2111–2114, 1985.
22. Tan G, Aw T, Dunstan R, Lee S: Evaluation of high performance liquid chromatography for routine estimation of hemoglobin A_2 and F. J Clin Pathol 46:852–856, 1993.
23. Maier-Redelsperger M, Noguchi CT, de Montalembert M, Rodgers GP, Schechter AN, Gourbil A, Blanchard D, Jais JP, Ducrocq R, Peltier JY, Cottat MC, Lacaille F, Belloy M, Elion J, Labie D, Girot R: Variation in fetal hemoglobin parameters and predicted hemoglobin S polymerization in sickle cell children in the first two years of life: Parisian prospective study on sickle cell disease. Blood 84:3182–3188, 1994.
24. Sutton M, Bouhassira EE, Nagel RL: Polymerase chain reaction amplification applied to the determination of β-like globin gene cluster haplotypes. Am J Hematol 33:66–69, 1989.
25. Périchon B, Ragusa A, Lapouméroulie C, Romand A, Moi P, Ikuta T, Labie D, Elion J, Krishnamoorthy R: Inter-ethnic polymorphism of the β-globin gene locus control region (LCR) in sickle cell anemia patients. Hum Genet 91:464–468, 1993.
26. Ragusa A, Frontini V, Lombardo M, Amata S, Lombardo T, Labie D, Krishnamoorthy R, Nagel RL: Presence of African beta-globin gene cluster haplotype in normal chromosomes in Sicily. Am J Hematol 40:313–315, 1992.
27. Bosze Z, Hans-Jurgen T, Charnay P: A transcriptional enhancer with specificity for erythroid cells is located in the long terminal repeat of the Friend's murine leukemia virus. EMBO J 5:1615–1623, 1986.
28. Kadonaga JT, Carner KR, Masiarz FR, Tijan R: Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell 51:1079–1090, 1987.
29. Talbot D, Philipsen S, Fraser P, Grosveld F. Detailed analysis of the site 3 region of the human β-globin dominant control region. EMBO J 9:2169–2178, 1990.
30. Elion J, Berg PE, Lapouméroulie C, Trabuchet G, Mittelman M, Krishnamoorthy R, Schechter AN, Labie D: DNA sequence variation in a negative control region 5′ to the β-globin gene correlates with phenotypic expression of the β^S mutation. Blood 79:787–792, 1992.